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# SYNOPSIS

## Machine Learning and Pharmacophore-based Virtual Screening of AgrA Inhibitors and *In Vitro* Evaluation of Antibiofilm Activity of Ellagic acid and its Conjugate against *Staphylococcus aureus*

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**OCTOBER 2024**

## Introduction

The spread of antibiotic resistance, which poses a grave threat to public health, has been caused by increased antibiotic use and consumption (Christaki *et al.,* 2020). However, infections caused due to antimicrobial resistance exhibited by bacteria can be acquired, adaptive, and intrinsic (Lee, 2019). Infections caused by multidrug-resistant bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA), have been associated with remarkable morbidity and mortality (Liu *et al.,* 2021). According to the 2019 Global Burden of Disease Study, *Staphylococcus aureus* (*S. aureus*) was the leading bacterial cause of death in 135 countries and was associated with the greatest number of deaths worldwide among people aged 15 and older (GBD, 2019).

*S. aureus* is a gram-positive pathogen that causes a variety of infectious diseases in humans and animals. These diseases range from minor infections of the skin and soft tissues to more serious and potentially fatal conditions like blood infections (bacteremia/septicemia). Biofilm, an extracellular polymeric substance (EPS) secreted by *S. aureus*, aids the microbe in resisting and minimizing the effects of antibiotics (Idrees *et al.,* 2021). Biofilm formation is controlled by the accessory gene regulator (Agr) quorum sensing system, and biofilm-producing *S. aureus* is less sensitive to conventional antibiotics than free-living planktonic cells (Ganesh *et al.,* 2022). Accordingly, there has been great interest in developing inhibitors of the Agr response. By regulating virulence factors, biofilm formation, and the heterogeneous resistance of MRSA, the Agr system plays a crucial role in pathogenesis.

When an autoinducer peptide (AIP) binds to the membrane- bound AgrC receptor, the Agr system is typically activated. The activated AgrC transfers a phosphate group to the response regulator AgrA. As a transcription factor, phosphorylated AgrA initially binds to the P2 promoter, which upregulates the Agr operon and amplifies the QS signal in a conventional autoinduction cycle, a trademark of QS systems. On the other hand, AgrA binds to the P3 and other promoters. The Agr-system is crucial to the expression of several virulence factors, including PSM production, which is associated with *S. aureus* pathogenicity. It upregulates the expression of virulence factors including enterotoxins, leukocidins, lipases, and proteases (Lade *et al.,* 2022).

The response regulator AgrA protein comprises two domains N-terminal CheY-like receiver domain (residues 1–130) and the C-terminal DNA binding domain (DBD) (residues 138–238). AgrADBD belongs to the LytTR protein family and binds to the consensus DNA elements in the P2 and P3 promoter regions. According to reports, this domain is found in all known *S. aureus* strains, including MRSA. the inhibition of AgrA binding to DNA reduces the

number of antibiotic-resistant strains and the capacity of bacteria to become pathogenic (Rajashree *et al.,* 2016; Iobbi *et al.,* 2021).

AgrA has an identical sequence in all four *S. aureus* subgroups, which makes it a more appropriate target than AgrC. In 2012, Leonard (Leonard *et al.,* 2012) proved that screening the small molecule database led to more *S. aureus* virulence inhibitor discoveries. Wang and Muir (2016) (Wang and Muir, 2016) inform that the biochemical analysis reveals that the small molecule blocks AgrA from binding to DNA.

Over the last two decades, the increasing number of active pharmaceutical ingredients with poor solubility has become one of the major challenges in pharmaceutical research. Most drug candidates from drug discovery programs indeed have poor water solubility. Approximately 40% of currently available drugs and 75% of drug candidates are estimated to have low water solubility (Nyamba *et al.,* 2021). Ellagic acid (EA) is a polyphenol, a dimeric derivative of gallic acid. The molecular formula of EA is C14H6O8 and is widely distributed in fruits such as pomegranates and berries such as strawberries, blackberries, and raspberries (Yu *et al.,* 2021). It possesses strong anti-oxidant, anti-inflammatory, anti-viral, anti-microbial, anti-teratogenic, hepatoprotective, and anti-tumor properties, particularly for colon cancer, esophageal cancer, breast cancer, and hepatocellular carcinoma (Yu *et al.,* 2023).

Ellagic acid is one of the active pharmaceutical ingredients with poor solubility. So, the therapeutic application of EA is limited due to its solubility in aqueous and other organic solvents, gastrointestinal tract permeability (GIT) and difficulties with the first-pass effect and irreversible binding to DNA and proteins (Hallan *et al.,* 2020). Therefore, more attention should be paid to overcoming this hurdle. So, numerous efforts have been made to address the issue. New formulations have been created using micronization, nanoparticles, powder form, inclusion complexes, encapsulation, emulsions, and combinations with other bioactive compounds (Zuccari *et al.,* 2020).

The drug discovery process can be speeded up using pharmacophore models used as the query to search compound libraries to pull out molecules of interest with desired properties. Indeed, pharmacophore-based methods are widely used tools in CADD and are of great interest in the chemo-informatics field since they find many applications in drug discovery projects, including not only virtual screening but also scaffold hopping, lead optimization, ligand profiling, target identification, multi-target drug or de novo drug design (Giordano *et al.,* 2022). Computer-aided drug design (CADD), including molecular docking, molecular dynamics simulations, QSAR analysis, and machine learning, are employed to predict the efficacy of

potential drug compounds and pinpoint the most auspicious compounds for subsequent testing and advancement (Iwaloye *et al.,* 2023).

Despite various attempts to discover a lead molecule with antibiofilm and anti quorum sensing activities, bacterial resistance still grows alarmingly. With this background, the present study focused on identifying potential inhibitors with antibiofilm and anti-quorum sensing properties. It could serve as a molecule that can act as a lead for the design of a novel antibiofilm agent against AgrA in *Staphylococcus aureus*. The present research work was designed with the following objectives:

- ✓ To identify potent inhibitors of AgrA against *Staphylococcus aureus* using machine learning, Pharmacophore, and Molecular Dynamics approaches.
- ✓ To determine the anti-biofilm and antivirulence activity of the ellagic acid against *Staphylococcus aureus.*
- ✓ To synthesize and characterize gelatin-ellagic acid conjugate.
- ✓ To determine the antibiofilm ability of the synthesized conjugate coatings.

**Phase I: Screening of AgrA inhibitors from natural compound databases using machine learning, pharmacophore, and molecular dynamics approaches**

The 26 known inhibitors of AgrA were collected from the ChEMBL database and Literature, along with their corresponding IC50 values. The current study involves a two-step virtual screening process by combining machine learning and pharmacophore modeling. 1444 1D and 2D molecular descriptors were computed using ChemDes software. The inappropriate features were removed on the pre-processing of the dataset, and the number of descriptors decreased to 1018. To build an effective model, feature selection is a crucial process since this improves classification accuracy and reduces computing costs. In Weka software, the feature selection methods were employed. CfsSubset employs various search methods, including BestFirst, GreedyStepwise, PSOSearch, and WrapperSubset attribute evaluator using PSOSearch method. CfsSubset_BestFirst and CfsSubset_GreedyStepwise feature selection resulted in identical attributes and it is designated as set A. On the other hand, set B consisted of attributes from CfsSubset_PSOSearch (355), whereas set C contained attributes from the WrapperSubset_PSOSearch (71) method.

Four different machine-learning-based classification algorithms, namely, ANN, NB, RF, and SVM, were used for the development of various classification models with three

different feature sets. As a result, twelve different classification models were built. The ROC values of the classification models ranged between 0.59 and 0.885. In the case of ROC also the SVM model with set A datasets showed the greatest value of 0.885. Hence, the optimized SVM_Set A classification model resulting from the tenfold cross-validation method was selected for further prediction. After optimization selected SVM_Set A classification model has shown good values of MCC and ROC when validated via both tenfold CV and external validation.

The pharmacophore hypothesis was developed among them, the AAAH_1 pharmacophore hypothesis was selected for further screening process as it has the highest phase hypo score, EF1%, BEDROC160.9, and ROC. The best-fitted pharmacophore model AAAH_1 was found to have four pharmacophoric features, namely, three hydrogen bond acceptors (A3, A5, A6) (spheres with arrows indicated in red) and one hydrophobic feature (H12).

Potential AgrA inhibitors were retrieved by virtual screening approach wherein the selected pharmacophore hypothesis AAAH_1 was taken as the query in ZINC and COCONUT databases using ZincPharmer and Pharmit servers, respectively. ZINC is a free database of commercially-available compounds for virtual screening, with over 230 million purchasable compounds in 3D formats and 750 million available for search. It allows downloads by vendor and criteria like Lipinski-compliant, lead-like, and fragment-like compounds. The Zinc Natural Product database has over 85,000 natural products, separated by origin. The COCONUT database is an aggregated dataset of elucidated and predicted natural products from open sources, with a web interface for browsing and downloading. Virtual screening opted out hits that most perfectly correlated with the pharmacophoric features of the pharmacophore model. As a result, a total of 4992 compounds were retrieved as hits at the end of the screening process, of which 642 compounds were from the ZINC database and 4350 compounds were from the COCONUT database.

The 1D and 2D descriptors of the hit compounds extracted from ZINC and COCONUT databases were calculated, and their activity was predicted using the SVM model. Among the 4992 compounds, 3539 were finally screened out as active compounds. A molecular docking study of these compounds was performed to confirm their interaction with AgrA. These top seven compounds were found to fit on the active site of AgrA, with XP docking scores ranging between − 10.75 and − 7.0 kcal/mol. These compounds had good docking scores and amino acid interaction with AgrA. Molecular dynamics studies revealed that C1 and C6 were stable during the simulation period of 100 ns. Other compounds are moved away from the binding pocket of AgrA. ADMET analysis predicted that the C1 molecule has two Lipinski violations

and poor oral bioavailability, and C6 had no Lipinski violations. C1 is an ellagic acid (EA) derivative and C6 ellagic acid. EA is a polyphenolic compound present in fruit and berries. EA is a multitarget compound that has wound-healing, antibacterial, and antiviral effects, as well as acts as a systemic antioxidant. The low bioavailability greatly limits the clinical applications of EA due to the chemical nature of the organic hetero tetracyclic compound. Chemical structure modifications by esterification, encapsulation within nano-microspheres that serve as carriers, and molecular dispersion in polymer matrices are effective ways to improve EA's poor oral bioavailability. Further studies are needed to enhance the oral availability of EA and its derivatives, warrant the development of a novel and beneficial antibiofilm agent for *S. aureus.*

**Phase II: Determination of the anti-biofilm and antivirulence activities of the ellagic acid against *Staphylococcus aureus***

Ellagic acid was slightly soluble in Dimethyl sulfoxide (DMSO) (6.6mg in 10ml DMSO). Hence, the lowest concentrations of ellagic acid were assessed for its antibiofilm efficacy against *Staphylococcus aureus*. The growth of the culture was assessed at concentrations ranging from 2.0 – 16.5 µg/ml in Tryptic soy broth medium. Ellagic acid concentrations 2.0 – 16.5 µg/ml had no significant impact on *Staphylococcus aureus* growth. Therefore, 4,8,12 µg/ml concentrations of ellagic acid were used in subsequent studies. The EA exhibited concentration-dependent inhibition in *S. aureus*, which was calculated using a crystal violet assay. At 12 µg/ml concentration, EA inhibited a maximum of 48% biofilm formation of *Staphylococcus aureus.* In comparison, 4 and 8 µg/ml concentrations of EA attenuated the biofilm formation up to 30% and 41%, respectively. Ellagic acid effectively disrupted the preformed biofilm with 16% and 49% at 4 and 8 µg/ml, respectively. A reduction of 56% was observed at 12 µg/ml. The compound showed the ability to destroy preformed *Staphylococcus aureus* biofilms in a dose-dependent manner.

Biofilm formation was reduced in a dose-dependent manner. It was observed around the walls of test tubes at 4, 8 and 12 µg/ml concentrations of ellagic acid compared to the untreated control. Compared with untreated *Staphylococcus aureus,* ellagic-treated bacteria were highly dispersed and showed no apparent aggregates. The percentage of inhibition of carbohydrates observed at 4, 8 and 12 µg/mL were 20.98 %, 29.61%, and 32.55%, respectively. The protein content of *Staphylococcus aureus* biofilm decreased up to 12.24%, 14.29%, and 32.65%, respectively, upon EA treatment. On the other hand, EA treatment reduced lipid content by 32.3%, 40% and 48.4% at concentrations of 4, 8 and 12 µg/mL, respectively. Further, Agarose gel electrophoresis divulged EA's ability to reduce biofilm's

extracellularDNA content compared to untreated control. The compound at 4, 8, and 12 µg/mL reduced the diameter of the swarm zone from 10.73±0.63mm in untreated control to 8.23±0.25 mm, 7.63±0.25mm, and 5.73±0.49mm, respectively.

The percentage of metabolically active cells in biofilm was 46.69%, 31.44% and 27.33% after treatment with 4, 8, and 12 µg/mL. On treatment with 4, 8, and 12µg/mL, production of staphyloxanthin was decreased to 34.5%, 54.66% and 63.6%, respectively. The antibiotic gentamycin showed a staphyloxanthin inhibition percentage 75%which was higher than the 12 µg/mL of ellagic acid.

The decrease in protease activity with the halo zone (mm) from 10.23±0.25 8.63±0.25 to 6.40±0.29 on treatment with 4, 8, and 12µg/mL of ellagic acid, respectively. The lipase production inhibition absorbance at 400 nm was reduced to 1.07±0.02, 0.938±0.04, and 0.7243±0.01 for 4, 8, and 12µg/mL doses of ellagic acid, on comparison with untreated control (1.41±0.02).

The treatment with 4, 8, and 12µg/mL remarkably reduced the biofilm formation on surface glass coverslips. The FESEM analysis results also revealed that the ellagic acid 12µg/mL concentration reduces the biofilm formation compared to the untreated control. Thus, the results of Phase II demonstrated that the ellagic acid possesses good antibiofilm activity against *Staphylococcus aureus* and could serve as an antibiofilm agent against *Staphylococcus aureus.* The medicinal use of EA is limited due to the solubility in water and other organic solvents. Therefore, New formulations have been created using micronization, nanoparticles, inclusion complexes, encapsulation, emulsions, conjugation, and combinations with other bioactive compounds. So, Phase III led to preparing an ellagic acid inclusion complex and improving its water solubility.

**Phase III : Synthesis and characterization of gelatin-ellagic acid conjugate**

Gelatin-ellagic acid conjugate was synthesized by the free radical grafting method. The solubility of EA in GEA conjugate was 0.344 µg/ml, whereas the unconjugated EA was 0.229µg/ml. Therefore, the solubility was found to be increased by 15-fold. Plackett-Burman (PB) design is a well-established and widely used statistical design for screening and selecting media components. The conjugate synthesis was optimized using Plackett–Burman Design on Minitab 17 software. The six factors gelatin, ascorbic acid, hydrogen peroxide, pH, temperature and incubation time were selected for PB design. The data on grafting efficiency was subjected

to multiple linear regression analysis. The model generated using the Plackett- Burman design was found to have an $R^2$ value of 99.46%, indicating a good correlation between observed and predicted response. The Adj $R^2$ of 98.97% indicated that the model would have a strong association even with adding extra variables. The analysis of variance showed that the model was significant, with a model F-value of 761.85. The factors with $p < 0.05$ affected the grafting efficiency significantly. Gelatin, ascorbic acid, and hydrogen peroxide have p values less than 0.05, which indicates that they significantly affect the grafting efficiency. Among them, ascorbic acid, temperature and incubation time, which show positive coefficients, indicated their positive effect on grafting efficiency.

Pareto analysis demonstrated that the concentration of Hydrogen peroxide is significant, followed by H and L as Dummy variables. Ascorbic acid and gelatin are of greater significance for the grafting efficiency of ellagic acid into gelatin. In contrast, pH, temperature, and incubation time were non-significant. Statistical analysis of the Plackett-Burman design shows that the gelatin and hydrogen peroxide had negative coefficients, and ascorbic acid only had a positive coefficient. The final parameter chosen based on all the analyses mentioned above was ascorbic acid, which significantly affects the grafting efficiency of ellagic acid into gelatin. The results of single-factor experiments, the maximum grafting ratio (167.67 µg ellagic acid/mg gelatin grafted ellagic acid conjugate) was obtained at 24 h with 0.121mg/ml ellagic acid, GA, two g/L ascorbic acid and 0.2 M hydrogen peroxide. UV, FTIR and 1HNMR analysis revealed that the ellagic acid was successfully grafted into gelatin. The results of Phase III indicated that the gelatin ellagic acid conjugate was successfully synthesized and characterized.

**Phase IV: Antibiofilm ability of free and surface coated gelatin-ellagic acid conjugate against *Staphylococcus aureus***

The fourth phase of the study evaluated the effect of gelatin ellagic acid conjugate against MRSA and formulated an effective polymeric antibiofilm coating for implant applications. Initially, the growth of the culture was assessed at concentrations ranging from 16.45 – 41.13 µg/ml of the ellagic acid content of conjugate in a TSB medium. The results showed that 16.45 – 41.13 µg/ml concentrations had no significant impact on *Staphylococcus aureus* growth. At 41.13 µg/ml concentration, EA inhibited a maximum of 73% biofilm formation of *Staphylococcus aureus.* In comparison, 16.45, 24.68, and 32.90 µg/ml concentrations of EA attenuated the biofilm formation up to 59%, 61%, and 64%, respectively.

A coating-based approach is emerging to prevent bacteria adhesion on biomaterials. Active coatings containing antibiofilm agents are being developed to combat biomaterial-associated infections. The antibiofilm coating with ellagic acid gelatin conjugate as an active ingredient was formulated and coated on the glass surface by screen printing. Various concentrations of ellagic acid ranging from 16.7 – 83.5 µg in conjugate were coated on the glass slides and assessed for their antibiofilm activity against *Staphylococcus aureus*. The biofilm inhibition was observed in the well, and a few isolated colonies were obtained after imprinting the slides on a nutrient agar plate. No growth on nutrient agar plates imprinted with slides was observed at 83.5µg of ellagic acid in conjugate.

**Conclusion**

The present study has provided a unique strategy to improve the bioavailability of ellagic acid, thereby combating *Staphylococcus aureus* infections.

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